INFECTION AND IMMUNITY, Dec. 2005, p. 8167–8178 Vol. 73, No. 12
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.12.8167–8178.2005

Iron and Fur Regulation in *Vibrio cholerae* and the Role of Fur in Virulence

Alexandra R. Mey,[1] Elizabeth E. Wyckoff,[1] Vanamala Kanukurthy,[1] Carolyn R. Fisher,[1] and Shelley M. Payne[1,2]*

Section of Molecular Genetics and Microbiology[1] and Institute for Cellular and Molecular Biology,[2] The University of Texas at Austin, Austin, Texas 78712-1095

Received 10 August 2005/Returned for modification 10 September 2005/Accepted 19 September 2005

Regulation of iron uptake and utilization is critical for bacterial growth and for prevention of iron toxicity. In many bacterial species, this regulation depends on the iron-responsive master regulator Fur. In this study we report the effects of iron and Fur on gene expression in *Vibrio cholerae*. We show that Fur has both positive and negative regulatory functions, and we demonstrate Fur-independent regulation of gene expression by iron. Nearly all of the known iron acquisition genes were repressed by Fur under iron-replete conditions. In addition, genes for two newly identified iron transport systems, Feo and Fbp, were found to be negatively regulated by iron and Fur. Other genes identified in this study as being induced in low iron and in the *fur* mutant include those encoding superoxide dismutase (*sodA*), fumarate dehydratase (*fumC*), bacterioferritin (*bfr*), bacterioferritin-associated ferredoxin (*bfd*), and multiple genes of unknown function. Several genes encoding iron-containing proteins were repressed in low iron and in the *fur* mutant, possibly reflecting the need to reserve available iron for the most critical functions. Also repressed in the *fur* mutant, but independently of iron, were genes located in the *V. cholerae* pathogenicity island, encoding the toxin-coregulated pilus (TCP), and genes within the *V. cholerae* mega-integron. The *fur* mutant exhibited very weak autoagglutination, indicating a possible defect in expression or assembly of the TCP, a major virulence factor of *V. cholerae*. Consistent with this observation, the *fur* mutant competed poorly with its wild-type parental strain for colonization of the infant mouse gut.

Iron is one of the most abundant elements in the earth's crust; however, iron is poorly soluble at physiological pH in the presence of oxygen and not readily bioavailable. Iron is essential for nearly all living organisms and is required for processes ranging from the tricarboxylic acid (TCA) cycle to electron transport, DNA metabolism, and response to oxidative stress. Iron can also be detrimental to cells, due to the reactive oxygen species produced in the presence of this element. Thus, the influx and intracellular processing of iron is tightly regulated. In many bacterial species, this regulation is carried out by the iron-dependent negative regulator Fur, which coordinates the level of intracellular iron with expression of genes involved in iron uptake, storage, and metabolism. When iron is abundant, Fur complexes with ferrous iron and blocks transcription of target genes by binding to conserved promoter regions termed Fur boxes. In *Escherichia coli*, the Fur box has been described as a 19-bp consensus sequence encompassing two staggered motifs on opposite faces of the DNA helix, to which Fur may bind as a dimer of dimers (2).

Most genes involved in iron acquisition are repressed by the Fur-Fe^{2+} complex, thus ensuring that these genes are expressed only when the level of free iron in the cell is low. In contrast, several genes involved in iron storage, iron metabolism, and antioxidant defense appear to be positively regulated by Fur and iron (16, 52, 60), and this has been shown to involve a variety of mechanisms. In *Helicobacter pylori*, *sodB*, encoding superoxide dismutase, and *pfr*, encoding a non-heme-containing ferritin, are repressed by Fur in the absence of iron (14, 18). When complexed with iron, Fur cannot bind to the operator sequences of these genes, and the repression is relieved (14, 18). Direct activation of gene expression by Fur has been reported as well. In *Neisseria meningitidis*, transcription of the nitric oxide reductase-encoding gene *norB* is activated in the presence of iron by the direct binding of Fe^{2+}-Fur to a Fur box sequence in the *norB* operator (13). Most cases of positive regulation by Fur reported to date, however, involve the action of a small RNA, RyhB (43). In *E. coli*, RyhB negatively regulates the expression of *sodB*, *ftn* and *bfr* (ferritin and bacterioferritin), and several iron-sulfur cluster-containing TCA cycle enzyme genes, including the *sdh* operon (succinate dehydrogenase) and *acnA* (aconitase). Because RyhB is itself repressed by Fur, the net result is positive regulation of these genes under conditions of high iron. In this way, while iron limitation results in increased synthesis of iron acquisition proteins, iron abundance stimulates production of iron-containing proteins and promotes sequestering of iron within ferritin and bacterioferritin complexes.

Vibrio cholerae, the causative agent of the diarrheal disease cholera, requires iron for growth and possesses a variety of iron acquisition systems. *V. cholerae* synthesizes and secretes the catechol siderophore vibriobactin, a high-affinity iron chelator that scavenges extracellular iron and facilitates its transport into the cell (27). In addition, *V. cholerae* has transport systems for siderophores made by other microorganisms, including enterobactin (49, 82) and ferrichrome (27, 63). Heme and hemo-

* Corresponding author. Mailing address: The University of Texas, Section of Molecular Genetics and Microbiology, Austin, TX 78712-1095. Phone: (512) 471-9258. Fax: (512) 471-7088. E-mail: payne@mail.utexas.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
V. cholerae strains		
O395	*V. cholerae* classical biotype	46
ARM572	O395 *ryhB*Δ::*kan*	47
ARM573	O395 *fur*Δ::*tmp*	47
N16961	*V. cholerae* EI Tor biotype	J. Kaper
E. coli strains		
DH5α	Cloning strain	28
DH5α(λ*pir*)	Host strain for pHM5 derivatives	J. Kaper
Plasmids		
pHM5	Suicide vector carrying *sacB*; Cb^r Suc^s	64
pQE-2	IPTG-inducible expression vector	QIAGEN
pWKS30	Low-copy cloning vector; Cb^r	75
pAMF1	*V. cholerae fur* in pWKS30	This study
pAMR70	*V. cholerae ryhB* in pQE-2	47
pAMS17	*V. cholerae fur* in pHM5	This study

globin are sources of iron for *V. cholerae*, and this is reflected in the multiple transport systems dedicated to heme (34, 35, 48, 53, 67). Finally, genes encoding two separate systems for the transport of inorganic iron, *feo* and *fbp*, are present in the *V. cholerae* genome (33; E. E. Wyckoff, unpublished data).

Negative regulation by both Fur and iron has been demonstrated experimentally for several of the *V. cholerae* iron acquisition systems (8, 34, 42, 63, 69), and it is anticipated that most, if not all, of these systems are Fur and iron repressed. Other regulatory patterns involving iron and Fur have also been observed in *V. cholerae*. Analysis of protein expression profiles by two-dimensional gel electrophoresis suggested positive, as well as negative, regulation by iron and Fur and both positive and negative regulation by iron, independently of Fur (42). As was shown for *E. coli* (30), a *fur* mutant of *V. cholerae* could not use pyruvate, succinate, and fumarate as carbon sources, suggestive of defects in particular steps of the TCA cycle (42). We (47) and others (12) have recently reported the characterization of *V. cholerae* RyhB, a functional homolog of *E. coli* RyhB. *V. cholerae* RyhB, which is negatively regulated by iron and Fur, represses the expression of many TCA cycle genes, consistent with the observed carbon source utilization defects of the *fur* mutant. Interestingly, we found no evidence that *V. cholerae* RyhB controls expression of genes encoding ferritin or bacterioferritin, as is reported in *E. coli*. Thus, while some aspects of Fur and RyhB regulation are well conserved between these two species, others appear to be unique to one or the other. In this study we report the characterization of the Fur and iron regulons in *V. cholerae* and demonstrate a role for Fur in *V. cholerae* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. All strains were maintained at −80°C in tryptic soy broth plus 20% glycerol. Strains were routinely grown at 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) (50) or on LB agar. For microarray analyses, strains were grown in EZ rich defined medium (EZ RDM; http://www.genome.wisc.edu/functional/protocols.htm), a modification of the supplemented morpholinepropanesulfonic acid-defined medium described by Neidhardt et al. (51). For iron-limited EZ RDM, iron was omitted from the basic medium, and for iron-replete EZ RDM, ferrous sulfate ($FeSO_4$) was added to a final concentration of 40 μM. Sucrose (0.2% final concentration) was added to EZ RDM as the carbon source. Antibiotics were used at the following concentrations: for *E. coli*, 250 μg of carbenicillin per ml, 50 μg of kanamycin per ml, and 30 μg of chloramphenicol per ml; for *V. cholerae*, 125 μg of carbenicillin per ml, 25 μg of kanamycin per ml, 7.5 μg of chloramphenicol per ml, and 75 μg of streptomycin per ml. Inducible expression from plasmid pAMR70 was achieved using 200 μM isopropyl-β-D-thiogalactoside (IPTG). Electroporation of *V. cholerae* strains was carried out as described previously (53).

PCR. The oligonucleotide primers for PCR were purchased from IDT Inc. (Coralville, IA). PCR was performed using *Taq* polymerase (QIAGEN) or *Pfu* or Platinum *Pfx* polymerase (Stratagene) according to the manufacturers' instructions. Bacterial cultures grown overnight were used as the templates. All clones derived from PCR fragments were verified by sequencing.

Sequence analysis. DNA sequencing was performed by the University of Texas Institute for Cellular and Molecular Biology DNA Core Facility using an ABI Prism 3700 DNA sequencer. Analysis of DNA sequences was carried out using MacVector 7.1 and Clone Manager 7.04. The *V. cholerae* Fur box consensus sequence was built in WebLogo at http://weblogo.berkeley.edu/logo.cgi (65), using predicted Fur binding sequences of *V. cholerae* genes known to be involved in iron acquisition or metabolism as the training set.

Construction of plasmids and chromosomal mutants. To clone *V. cholerae fur* for complementation studies, the *fur* gene was amplified by PCR from strain O395 using Platinum *Pfx* polymerase and primers fur5 (5′-TTGGATTGCTTT GTGCCGAC) and fur6 (5′-TCCGTTACGACTACGACATTCCTC). The PCR product was digested with ClaI and cloned into pWKS30 digested with ClaI to create plasmid pAMF1. The *fur* mutation of ARM573 (47) was repaired by replacing the mutated allele with wild-type *fur* as follows: an EcoRV/SalI fragment containing the wild-type *fur* gene was excised from pAMF1 and cloned into pHM5 digested with EcoRV and SalI. The resulting plasmid, pAMS17, was transferred to ARM573 by bacterial conjugation, and allelic exchange was carried out as described previously (48) to create ARM574.

Microarray analysis. The *V. cholerae* microarray slides were generated in the Microarray Facility at the University of Texas at Austin as described elsewhere (47). For analysis of the *V. cholerae* Fur regulon, strains O395 and ARM573 were grown to an optical density at 650 nm of 0.5 in iron-replete EZ RDM. To study the iron-regulated transcriptome of *V. cholerae*, O395 was grown to an optical density at 650 nm of 0.3 in iron-replete or iron-depleted EZ RDM. Total RNA was extracted from an equal number of test and reference strain cells using RNeasy midi columns (QIAGEN). Using 15 μg of total RNA per sample, cDNA was synthesized in the presence of amino-allyl deoxyuridine triphosphates by reverse transcription with SuperScript II reverse transcriptase (Invitrogen) as per the manufacturer's instructions. Cy-3 fluorescent dye (Amersham Biosciences) was then coupled to the reference cDNA sample; Cy-5 was coupled to the test cDNA sample. The labeled cDNA samples were purified using the Mini Elute PCR purification kit (QIAGEN), and the cDNA probes were mixed and applied to the array surface for hybridization at 65°C for 4 h. Following hybridization, the arrays were washed, dried, and then scanned using a GenePix 4000 B scanner (Axon Instruments). The fluorescence intensities were determined using the GenePix Pro 4.2 software package. The Longhorn Array database (http://chipmunk.icmb.utexas.edu/ilat/) was used to perform data filtering and analysis (41). Only hybridized oligonucleotide spots that passed the quality control filters for minimum intensity and pixel consistency were included for further analysis. For each experimental condition, four independent arrays were performed. Differential expression was considered significant if, following normalization, the difference was ≥2-fold in at least two experiments and the average of all valid data points also met this criterion. The exception is Table 6, below, which lists genes within the mega-integron and the *tcp* cluster that had a ≥1.5-fold reduction in the mean expression ratio when comparing the *fur* mutant with the wild-type strain. The raw microarray data can be accessed at http://www.sbs.utexas.edu/paynelab/Public%20Array%20Data.html.

Autoagglutination assays. For autoagglutination assays (20), single colonies were resuspended in LB broth, and approximately 2×10^5 cells from this suspension were inoculated into 5 ml of low-salt LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at pH 6.5. Cultures were grown for 20 h on a rolling incubator at 30°C, and agglutination was evaluated visually after gently swirling the cultures and then allowing the aggregated cells to settle. To assess growth of the agglutinated cultures, the cultures were mixed vigorously and their optical densities were measured.

In vivo competition assays. In vivo competition assays were performed using 5-day-old BALB/c mice as described by Taylor et al. (71). Prestarved mice were inoculated intragastrically with 50 μl saline containing approximately 5×10^5 CFU of each competing strain grown to mid-log phase and 0.5% sucrose and

0.02% Evan's Blue dye. The mice were sacrificed after 24 h, and the small intestines were removed and homogenized in sterile phosphate-buffered saline. Serial dilutions were plated on medium selective for *V. cholerae* and then replica plated on differential medium to determine the viable counts for each competing strain. The output ratios were normalized to the input ratios to determine the competitive index (competitive index = [mutant ouput/wild-type ouput]/[mutant input/wild-type input]).

RESULTS

To characterize the genes regulated by iron and/or Fur in *V. cholerae*, microarray analysis was performed, comparing gene expression in the wild-type strain O395 grown with iron and O395 grown without iron supplementation and comparing the *fur* mutant with the parental strain. Both iron limitation and the *fur* mutation affected the growth rate of *V. cholerae*, and this was most evident during the late logarithmic and stationary growth phases (data not shown). To avoid differential gene expression due to growth rate differences, cell density was monitored during growth, and cultures were harvested during early to mid-logarithmic phase, when the strains to be compared were growing at the same rate.

Genes repressed by iron and Fur. The majority of genes differentially regulated in these analyses were derepressed in low iron and in the *fur* mutant (Table 2). As expected, many of these genes encode proteins involved in iron acquisition, including those required for biosynthesis of the siderophore vibriobactin and those responsible for transport of iron ligands into the cell (Table 2). All the siderophore receptor genes were highly induced in low iron and in the *fur* mutant (Table 2), and the heme receptor gene *hutA* was among the most iron- and Fur-responsive genes identified in this study (Table 2). In contrast, the *hasR* heme receptor gene was only weakly regulated and did not meet the criteria for inclusion in the data set (data not shown). The third heme receptor gene, *hutR*, was not itself demonstrably regulated by iron or Fur; however, the first gene of the *hutR* operon, *ptrB* (VCA0063), was significantly induced in low iron and in the *fur* mutant (Table 2). VC0284, encoding a putative outer membrane receptor, was regulated by both iron and Fur (Table 2). Although no ligand has been identified for this proposed receptor, preliminary studies suggest that it functions in iron acquisition (A. R. Mey, unpublished data). All of the known ABC transport systems for the transport of iron or iron complexes across the inner membrane were regulated (Table 2), including *fhuBD* and *fbpBC*, but these appeared to be only weakly iron responsive and thus did not pass the selection criteria (data not shown). Also regulated were homologs of the ferrous iron transport genes *feoAB*, as well as a small open reading frame (ORF) downstream of *feoB*, VC2076 (Table 2) (29).

Genes other than those involved in iron acquisition were also regulated by Fur and iron. These included *sodA*, encoding the manganese-containing superoxide dismutase, and *fumC*, the noniron form of fumarate hydratase (Table 2), as well as *bfr* and *bfd*, encoding, respectively, bacterioferritin and the bacterioferritin-associated ferredoxin (Table 2).

A number of genes of unknown function were negatively regulated by Fur and by high iron (Table 2). Some of these genes are closely linked to, and likely cotranscribed with, genes that are known to be iron regulated. It is not clear whether these genes have an as-yet-unidentified role in iron metabolism or if regulation by Fur is the fortuitous result of their location in the genome.

Nearly all of the genes involved in iron acquisition or metabolism have a potential Fur box sequence within the promoter driving their expression. A potential Fur box sequence for each of the genes with known functions in iron uptake and metabolism is listed in Table 3. These Fur box sequences were used to derive a consensus for the *V. cholerae* Fur box (Fig. 1), and this consensus sequence was in turn used to identify potential Fur boxes upstream of novel iron- and/or Fur-responsive genes found in this study (Table 4).

Among the newly identified genes upregulated in low iron and in the *fur* mutant (Table 2), only one, *irpA* (VC1264), has a proposed function in iron uptake or metabolism. In *Synechococcus* sp. strain PCC7942, the IrpA homolog is an iron-regulated membrane protein required for growth in low iron, but the biochemical function of this protein has not yet been established (62). In *V. cholerae*, two potential Fur boxes are located upstream of *irpA* (Table 4) (57), and the array analysis presented here confirms that it is iron regulated. The three ORFs of unknown function downstream of *irpA*, VC1265 to VC1267, were also induced in low iron and in the *fur* mutant (Table 2). It is unclear whether these downstream genes are cotranscribed with *irpA*. The start codon of VC1265 is approximately 200 bp downstream of VC1264, and a possible Fur box is located upstream of VC1265 (Table 4); however, no predicted transcriptional terminator is present in the intergenic region between these two genes, suggesting that there may be read-through from *irpA*.

An ABC transporter gene, VCA0977, for which no ligand has been described, was highly regulated by iron and Fur (Table 2). VCA0977 appears to be arranged in an operon with VCA0976, and a potential Fur box is located immediately upstream of these genes (Table 4). While VCA0977 has a wide phylogenetic distribution, homologs of VCA0976, encoding a hypothetical protein, are found only among the *Vibrionaceae*. Interestingly, all members of the *Vibrionaceae* that carry a homolog of VCA0976 also have the adjacent VCA0977 gene (data not shown).

Another iron- and Fur-repressed gene of unknown function is VCA0216 (Table 2). The two ORFs flanking VCA0216 were also repressed by iron, but not, apparently, in response to Fur (Table 2). VCA0215 to -217 are located on the small chromosome immediately adjacent to a region containing genes for a lipase, an extracellular protease, and a RyhB-regulated hemolysin (VCA0218 to VCA0223) (47, 54). It was proposed that this region may be part of a pathogenicity island that aids in the acquisition of iron and other nutrients by induction of host cell damage (54). Adjacent to this cluster are the *vct* genes, encoding transporters of enterobactin and catechol siderophores (VCA0227 to VCA0232) (49) (Table 2).

Genes induced by iron and Fur. A relatively small number of genes were positively regulated by iron and Fur under the conditions tested. Expression of the *nap* gene cluster, encoding an iron-rich periplasmic nitrate reductase complex that may function in the adaptation to anoxic conditions, was reduced in the *fur* mutant and in low iron (Table 2). Positive regulation of *nap* genes by Fur appears to be common, since similar regulation has been observed in *E. coli* (45), *Campylobacter jejuni* (36, 55), and *Pasteurella multocida* (58). No apparent Fur box

TABLE 2. Genes regulated by iron, with or without Fur

ORF[a]	Gene	Function[b]	*fur*/WT[c]	Low iron/high iron[d]
Negatively regulated by Fur and iron				
Vibriobactin biosynthesis				
VC0774	*vibA* (79)		8.5	9.7
VC0771	*vibB* (79)		11.7	10.1
VC0773	*vibC* (79)		11.0	14.7
VC0780	*vibD* (81)		2.7	ND[e]
VC0772	*vibE* (79)		8.7	11.0
VC2209	*vibF* (8)		7.7	10.6
VC0775	*vibH* (81)		3.0	5.6
Siderophore transport				
VC2211	*viuA*	OMT[f] vibriobactin (9, 66)	5.4	15.4
VC0475	*irgA*	OMT enterobactin (23, 25, 49)	32.5	18.8
VCA0232	*vctA*	OMT enterobactin (49)	11.3	14.3
VC0200	*fhuA*	OMT ferrichrome (63)	5.4	10.2
VC2210	*viuB*	Iron removal from catechol siderophores (7)	7.9	15.0
VC0776	*viuP*	PBP[g] catechols (82)	4.5	6.1
VC0777	*viuD*	IMT[h] catechols (82)	4.2	5.5
VC0778	*viuG*	IMT catechols (82)	4.2	15.6
VC0779	*viuC*	IMT catechols (82)	3.2	3.5
VCA0227	*vctP*	PBP[g] catechols (49)	4.8	8.9
VCA0228	*vctD*	IMT catechols (49)	2.3	4.6
VCA0229	*vctG*	IMT catechols (49)	2.3	4.1
VCA0230	*vctC*	IMT catechols (49)	3.7	7.9
VC0201	*fhuC*	IMT ferrichrome (63)	3.4	5.8
Heme transport				
VCA0576	*hutA*	OMT (34, 35, 48)	20.4	37.3
VCA0914	*hutB*	IMT (53)	10.4	ND
VCA0915	*hutC*	IMT (53)	2.7	5.8
VCA0916	*hutD*	IMT (53)	3.3	8.4
VCA0907	*hutZ*	Heme binding (80)	8.2	ND
Miscellaneous transporters				
VC0284		OMT ligand unknown[i]	3.6	5.3
VC2078	*feoA*	Ferrous iron[j]	3.8	4.1
VC2077	*feoB*	Ferrous iron[j]	3.6	5.2
VC0608	*fbpA*	Inorganic iron[j]	2.7	ND
TonB systems				
VCA0910	*tonB1* (53)		17.1	50.0
VCA0911	*exbB1* (53)		18.7	32.1
VCA0912	*exbD1* (53)		13.0	23.9
VC1544	*tonB2* (53)		7.1	6.2
VC1546	*exbB2* (53)		6.5	11.8
VC1545	*exbD2* (53)		6.0	7.6
Enzymes				
VC1542	*ligA*-2	Ligase (linked to *tonB2* operon)	2.2	2.4
VC1573	*fumC*	Fumarate dehydratase	9.1	13.8
VC2694	*sodA*	Superoxide dismutase	7.5	23.8
VCA0063	*ptrB*	Protease II (48)	4.9	7.3
Regulatory proteins				
VC0474	*irgB*	Regulator of *irgA* (24)	1.7	2.3
VCA0231	*vctR*	Linked to *vctA*, function unknown (49)	8.7	10.5
Iron storage				
VC0364	*bfd*		3.7	3.2
VC0365	*bfr*		3.7	3.2
Unknown function				
VC0091		Putative *O*-methyl transferase for polyketide biosynthesis, COG3315	4.8	4.4
VC1264	*irpA*	Function unknown, COG3487	5.4	10.0
VC1265		Putative thioloxidoreductase, linked to *irpA*, COG3488	3.1	3.0
VC1266		Hypothetical periplasmic lipoprotein, linked to *irpA*, COG3489	2.7	3.9
VC1267		Hypothetical, linked to *irpA*, COG3490	2.9	4.2
VC1543		Hypothetical, linked to *tonB2* (53)	3.9	4.1

Continued on following page

TABLE 2—*Continued*

ORF[a]	Gene	Function[b]	*fur*/WT[c]	Low iron/high iron[d]
VC1547		*exbB* related, linked to *tonB2* (53)	4.6	9.2
VC1548		Hypothetical, linked to *tonB2* (53)	7.9	13.3
VC1572		Hypothetical, linked to *fumC*	9.4	8.9
VC1688		Hypothetical	2.6	2.9
VC2076		Hypothetical, linked to *feoAB*	2.4	2.7
VC2695		rRNA methylase, linked to *sodA*, COG0219	2.7	5.0
VCA0043		Putative acyl transferase, COG2388	4.3	3.8
VCA0216		Hypothetical membrane, linked to VCA0215 and VCAϕ217	3.7	7.2
VCA0233		Hypothetical, linked to *vctA*, COG3553 (49)	6.0	5.7
VCA0234		Hypothetical, linked to *vctA* (49)	4.8	8.9
VCA0909	*hutW*	Unknown, linked to *hutZ* (80)	20.0	52.1
VCA0908	*hutX*	Unknown, linked to *hutZ* (80)	ND	51.3
VCA0976		Hypothetical	19.6	53.4
VCA0977		ABC transporter, linked to VCA0776, COG4172	12.7	20.5
VCA1041		Putative phosphomannomutase, COG1109	2.8	2.5
Positively regulated by Fur and by iron				
VCA0678	*napA*	Periplasmic nitrate reductase	0.44	0.18
VCA0679	*napB*	Periplasmic nitrate reductase	0.43	0.16
VCA0680	*napC*	Periplasmic nitrate reductase	0.58	0.30
VCA0677	*napD*	Maturation of nitrate reductase	0.43	0.16
VCA0676	*napF*	Periplasmic nitrate reductase	0.41	0.24
VC1973	*menB*	Menaquinone biosynthesis	ND	0.41
Negatively regulated by iron, independently of Fur				
VC1112	*bioB*	Biotin biosynthesis	0.86	3.6
VC2227	*purN*	Purine biosynthesis	1.0	2.8
VC2415	*pdhR*	Pyruvate dehydrogenase repressor	0.93	3.4
VCA0215		Hypothetical, linked to VCA0216	1.1	3.2
VCA0217		GGDEF motif protein, linked to VCA0216, COG2199	1.2	3.2
VCA0262		Hypothetical	0.95	3.2
Positively regulated by iron, independently of Fur				
VC1216		Hypothetical, GGDEF motif protein, COG2199	0.97	0.34
VC1343		Peptidase M20, COG2195	0.89	0.43
VC1371		Hypothetical	0.97	0.49
VC1514		Hypothetical	1.2	0.23
VC1515		Putative formate dehydrogenase-specific chaperone, COG3381	1.0	0.33
VC1516		Iron-sulfur cluster binding protein, COG1145	0.95	0.37
VCA0784		Putative reductase, COG3007	0.95	0.50

[a] ORF designation from TIGR database (33).
[b] The reference(s) for previously identified genes is indicated. For genes of unknown function, the cluster of orthologous groups (COG) (70) number is included if available.
[c] Mean expression ratio of *fur* mutant relative to wild-type parental strain O395.
[d] Mean expression ratio of wild-type strain O395 grown in the absence relative to the presence of iron supplementation.
[e] ND, not determined. Data for this gene did not pass the quality filters.
[f] OMT, outer membrane transporter.
[g] PBP, periplasmic binding protein.
[h] IMT, inner membrane transporter.
[i] A. R. Mey, unpublished data.
[j] E. E. Wyckoff, unpublished data.

was present upstream of the *V. cholerae nap* genes, and it is not known whether the effect of Fur on the expression of these genes is direct. The *menB* gene for the synthesis of menaquinone was also downregulated in low iron, but we did not obtain data for this gene in the array analysis of the *fur* mutant. Neither the *nap* genes nor *menB* was found to be part of the RyhB regulon (12, 47), suggesting that the positive effect of Fur on these genes may be independent of RyhB.

Genes regulated by iron independently of Fur. Only six genes had increased expression in low iron but not in the *fur* mutant (Table 2). One of these, *bioB*, is required for biotin biosynthesis. Genes for biotin biosynthesis are induced in response to low iron in a number of organisms; these include *bioA* in *C. jejuni* (36) and *bioB* in *Helicobacter pylori* (17). The *purN* gene, which encodes a purine biosynthetic enzyme, was also regulated in this manner, as were VCA0215 and VCA0217, genes of unknown function linked to the iron- and Fur-regulated ORF VCA0216.

The expression of seven genes was reduced in low iron but unchanged in the *fur* mutant (Table 2). Two of these, VC1515 and VC1516, appear to encode proteins involved in the assembly of formate dehydrogenase, and a third, VC1514, is linked

TABLE 3. Fur boxes of known iron acquisition or metabolism genes in *V. cholerae*[a]

Gene name	VC no.	Proposed Fur box sequence	Reference(s) for Fur box sequence
ryhB	Small RNA	TTAAATGAGAACTATTATT	12, 47
fhuA	VC0200	TTTAATTAAGATAATTATC	This study
(none given)	VC0284	GAAAATTACTATCATTTGT	57
(none given)	VC0284	ATCAATAATGAAAATTACT	This study
irgBA intergenic region	VC0474	GATAATTATTCTTAATTTC[b]	24, 76
fbpA	VC0608	GATAAAGGTTATCATTACT	This study
vibB	VC0771	GATAATTGTTATTATTTAC	This study
vibC	VC0773	GCAAATGTAATCTGTTGCG	This study
vibA	VC0774	ATAAATGCAAGCAATTATC	79
vibH-viuP intergenic region	VC0775	AATATTGATTCTCATTTCG	57
viuC	VC0779	CGGAATAAAGAGAATTTTG	82
VC1548 (*tonB2* operon)	VC1548	GATAATGAGAGCGTTTCTC	This study
fumC	VC1573	GATAATAATTATCATTTAA	This study
feoA	VC2078	AGTAATATTTCTTATTAAC	This study
vibF	VC2209	GATAATGATTATTATTAAC[b]	8, 57
viuB	VC2210	GTTAATGATATACATTCTC	7, 57
viuA	VC2211	GCAAATGAGAATGCTTTAC	9
viuA	VC2211	GTGAATTATTAAGATTCTC	9
sodA	VC2694	GTTAATGATATTAATTATC	This study
ptrB (*hutR* operon)	VCA0063	GATAATTGATCTTATTTAG	48
vctP	VCA0227	CTTAATGAGAATAAGTATC	57
vctA	VCA0232	ATATATGCGAATCGTTATC	57
hutA	VCA0576	ACAAATGATAGCAATTATC	34
hasR	VCA0625	GATAATAATTATCAATTGC	This study
hutZ	VCA0907	ATCATTAGCGTCAATTTAT	80
hutZ	VCA0907	CTTAATAATAGCAATTATC	80
tonB1	VCA0910	GATAATTGCTATTATTAAG	This study
tonB1	VCA0910	GCTAATGATAATGCAATTG	53

[a] A complete list of references for the genes listed here is given in Tables 2 and 5.
[b] Fur box sequence has been verified by DNase I footprinting.

to those genes. Like the Nap components, the products of this operon may be part of the response to anoxic conditions.

Genes regulated by Fur, but independently of iron. Seven genes were derepressed in the *fur* mutant but were not regulated in response to iron availability (Table 5). A striking member of this group is VC1562, which has homology to genes encoding Zn-dependent hydrolases of the β-lactamase family. Expression of VC1562 was increased more than 20-fold in the *fur* mutant, and a potential Fur box was identified, albeit within the upstream ORF. Another gene in this group is VCA0969, encoding a pirin-related protein belonging to the iron-containing cupin family, which has both prokaryotic and eukaryotic members (77). The putative cytochrome b_{561} gene, VCA0538, was also negatively regulated by Fur. Interestingly, the other *V. cholerae* cytochrome b_{561} ortholog, VCA0249, is negatively regulated by RyhB (47), suggesting that Fur may have opposite effects on these two genes.

All the genes that were repressed in the *fur* mutant inde-

FIG. 1. The *V. cholerae* Fur box consensus sequence. Predicted, as well as experimentally verified, Fur box sequences within the promoters of known iron acquisition and metabolism genes in *V. cholerae* (Table 3) were used as the training set to derive a consensus sequence for the *V. cholerae* Fur binding site. WebLogo (65) was used to build a consensus sequence logo, in which the height of individual letters within a stack of letters represents the relative frequency of that letter at a given position, and the overall height of the stack represents the degree of conservation at that position.

TABLE 4. Potential Fur boxes associated with newly identified Fur-regulated genes

Locus	Gene	Putative Fur box sequence
VC0091		GAGATTCATTATCATCTTC[a]
		CTTATTTGAGATTCATTAT[a]
VC0364	*bfd*	AGAAATAAGAGTGATTCTC[a]
		GTGATTCTCTTTTATTCTT[a]
VC0788		AGAAATCGACCTTATTGTG
VC1048		CTTAATGCGAGTGATTCTC
VC1264	*irpA*	ACAAATGATAATAATTTGC[a]
		GATAATAATTTGCAATTCA[a]
		GTAAATTTGTATTATTTGC
VC1265		TGCAATAAAACCCAATCTC
VC1562		GTTATTAAATTTAAATCAA[a,b]
		ATAAATTTAAATCAATGCT[a,b]
VC1572	*fumC* operon	GATAATAATTATCATTTAA
		TGCAATCAGCAGTAATGTG
VC1688		GATAATAATTCTCATTCAT[a]
		ATAATTCTCATTCATTGCG[a]
VC2694	*sodA*	GTTAATGATATTAATTATC[b]
VCA0216		ATAAATGAGAATTAATATC
VCA0969		CCCAATCATTCAAATTTCT
VCA0976		GGTAATTATTTGCATTTGA
		TAAATTGCATTTAAATATC
VCA1041		GAGAATAAATATCAATTAG

[a] This putative Fur box overlaps with another potential Fur box.
[b] The proposed Fur box is located within the coding region of the upstream gene.

TABLE 5. Genes negatively regulated by Fur but independent of iron

ORF[a]	Gene	Function	*fur*/WT[b]	Low iron/high iron[c]
VC0788		DOPA dioxygenase related, COG3805	2.8	1.0
VC1048		Hypothetical, COG0778	2.4	1.8
VC1461	*cep*	Colonization factor	2.1	1.3
VC1562		β-Lactamase-related protein, zinc hydrolase family, COG0491	21.6	1.2
VCA0538		Putative cytochrome b_{561}, COG3038	2.1	1.3
VCA0734		Hypothetical	2.9	0.99
VCA0969		Pirin-related protein, cupin family of iron binding proteins, COG1741	3.8	0.99

[a] ORF designation from TIGR database (33).
[b] Mean expression ratio of the *fur* mutant relative to wild-type parental strain O395.
[c] Mean expression ratio of the wild-type strain O395 grown in the absence relative to the presence of iron supplementation.

pendently of iron mapped in one of two gene clusters (Table 6). The first cluster is located within the mega-integron on the small chromosome (33, 44). The function of many of these genes is unknown, but the Fur-regulated gene products in this locus include a RelE type of toxin-antitoxin system (22, 56). The other group of genes maps within the *V. cholerae* pathogenicity island, which encodes proteins required for formation of the toxin-coregulated pilus, TCP, an essential virulence factor of *V. cholerae* (11, 71). Many of the genes within these clusters were repressed approximately 2-fold in the *fur* mutant; however, to give a more complete representation of the effect of the *fur* mutation, data for all the genes within the mega-integron and the *tcp* cluster that were repressed at least 1.5-fold were included in Table 6. The effect of Fur did not appear to be dependent upon iron, since no consistent pattern of regulation in response to iron levels was observed.

The *V. cholerae fur* mutant has a defect in autoagglutination. Decreased expression of the *tcp* genes in the Fur mutant suggested that Fur may be required for optimal expression of

TABLE 6. Genes within the large integron and the *tcp* region positively regulated by Fur

ORF[a]	Gene	Function	*fur*/WT[b]	Low iron/high iron[c]
Large integron				
VCA0444	*relE*	Toxin of toxin-antitoxin stress response locus, COG2026	0.48	0.83
VCA0445		Antitoxin of toxin-antitoxin stress response locus, COG2161	0.37	0.92
VCA0446		Hemagglutinin	0.43	0.91
VCA0451		Hypothetical	0.58	1.1
VCA0453		Hypothetical	0.56	1.1
VCA0455		Hypothetical, COG0693	0.63	0.94
VCA0457		Hypothetical	0.45	1.2
VCA0458		Hypothetical	0.39	0.96
VCA0459		Hypothetical	0.46	1.2
VCA0462		Hypothetical	0.74	1.1
VCA0466		Hypothetical	0.43	ND[e]
VCA0468		Hypothetical, COG4737	0.41	0.77
VCA0469		HLH_XRE transcriptional regulator family, COG2944	0.38	0.95
VCA0470		Putative acetyltransferase, COG0456	0.47	1.1
VCA0471		Hypothetical	0.54	0.90
TCP region[d]				
VC0819	*aldA-1*		0.44	1.2
VC0820	*tagA*		0.38	0.81
VC0821		Hypothetical	0.51	0.76
VC0822		Putative inner membrane	0.51	0.97
VC0823		Hypothetical	0.60	0.70
VC0824	*tagD*		0.53	0.56
VC0825	*tcpI*		0.48	0.93
VC0829	*tcpB*		0.45	0.76
VC0830	*tcpQ*		0.49	1.5
VC0831	*tcpC*		0.47	1.5
VC0832	*tcpR*		0.45	1.7
VC0833	*tcpD*		0.44	1.8
VC0834	*tcpS*		0.43	ND
VC0835	*tcpT*		0.57	1.5
VC0836	*tcpE*		0.43	1.2
VC0837	*tcpF*		0.22	1.0
VC0840	*acfB*		0.58	1.3
VC0841	*acfC*		0.55	1.3
VC0842		Hypothetical, COG1714	0.52	1.6
VC0843	*tagE*		0.54	1.1
VC0844	*acfA*		0.40	1.1
VC0845		Hypothetical	0.56	0.73
VC0846		Integrase, degenerate	0.75	1.0

[a] ORF designation from TIGR database (33).
[b] Mean expression ratio of *fur* mutant relative to wild-type parental strain O395.
[c] Mean expression ratio of wild-type strain O395 grown in the absence relative to the presence of iron supplementation.
[d] For a review of individual genes in the *tcp*/*acf* cluster, see reference 19.
[e] ND, not determined.

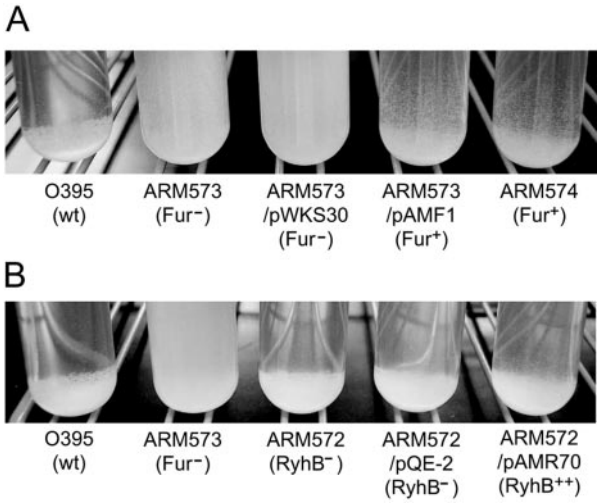

FIG. 2. The role of Fur in *V. cholerae* autoagglutination. Strains were inoculated into LB broth at pH 6.5 and grown on a rolling shaker at 30°C for 20 h. (A) O395 (wild type), ARM573 (*fur*Δ::*tmp*), ARM573/pWKS30 (vector control), ARM573/pAMF1 (complementing *fur* plasmid), and ARM574 (wild-type *fur* allelic replacement derivative of ARM573). (B) O395 (wild type), ARM572 (*ryhB*Δ::*kan*), ARM573 (*fur*Δ::*tmp*), ARM572/pQE-2 (vector control), and ARM572/pAMR70 (IPTG-inducible *ryhB* expression construct).

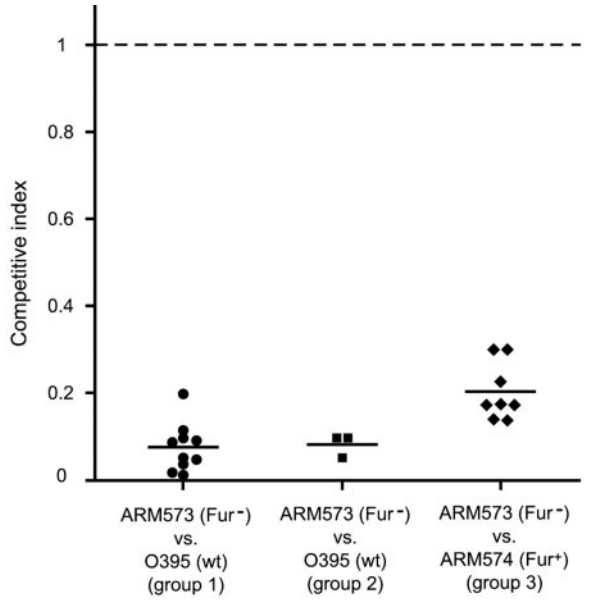

FIG. 3. Fur is required for efficient intestinal colonization. For each competition, the indicated strains were grown under the conditions described and coinoculated intragastrically into 5-day-old BALB/c mice, as detailed in Materials and Methods. The competitive index was calculated by normalizing the output ratio to the input ratio of the two competing strains. Each data point represents one mouse, and the average competitive index for each experimental group is represented by a horizontal line. A competitive index below 1 (shown by the dashed line) indicates that the *fur* mutant is at a competitive disadvantage. Three experimental groups are shown. Group 1 and group 3 strains were grown in LB broth, pH 7, at 37°C to mid-log phase prior to inoculation, while group 2 strains were grown in low-salt LB broth, pH 6.5, at 30°C (TCP-inducing conditions) to mid-log phase prior to inoculation.

these genes. Proper synthesis of the TCP correlates with the ability of *V. cholerae* to autoagglutinate under particular culture conditions, and strains carrying mutations in TCP structural genes (11, 71) or regulatory genes (10, 11, 20, 71, 74, 78) do not autoagglutinate. To test whether Fur plays a role in the autoagglutination of *V. cholerae*, the wild-type (O395) and *fur* mutant (ARM573) strains were grown under conditions known to promote *tcp* expression and autoagglutination. As shown in Fig. 2A, the agglutinated wild-type cells settled to the bottom of the tube, leaving the supernatant clear. The *fur* mutant cells, however, did not agglutinate efficiently, and the supernatant did not clear, suggesting defects in the expression or assembly of the TCP or some other factor(s) required for autoagglutination. The optical density of the *fur* mutant culture was similar to that of the wild-type culture (data not shown), indicating that the *fur* mutant did not have a growth defect under these conditions. The differences in autoagglutination between the wild-type and the *fur* mutant strains are therefore not likely to be caused by disparities in growth rate or cell density. The autoagglutination defect was complemented by supplying wild-type *fur* on the plasmid pAMF1, demonstrating that the autoagglutination defect is due to the loss of *fur* (Fig. 2A). Autoagglutination was also restored in strain ARM574, in which the mutated *fur* allele of strain ARM573 was replaced with a wild-type copy of *fur* by allelic exchange.

Fur has been shown to exert many of its effects indirectly, by repressing synthesis of the negative regulator RyhB. This presented the possibility that the autoagglutination defect of the *V. cholerae fur* mutant was due to deregulation of *ryhB* expression. To test the involvement of RyhB in autoagglutination, a *ryhB* mutant, ARM572, as well as ARM572 expressing high levels of *ryhB* from an inducible promoter on plasmid pAMR70 were tested in the agglutination assay (Fig. 2B). The results indicated that *V. cholerae ryhB* is not required for autoagglutination, nor does overexpression of *ryhB* inhibit the agglutination process. Thus, the defect of the *fur* mutant is not due to overproduction of RyhB in the absence of Fur.

***V. cholerae fur* plays a role in in vivo colonization.** Because autoagglutination of *V. cholerae* correlates with expression of the TCP and with virulence (71), the role of Fur in *V. cholerae* virulence was tested using the infant mouse model of intestinal colonization (71). An equal number of cells from the *fur* mutant (ARM573) and its parental strain (O395) were coinoculated intragastrically into 5-day-old infant mice, and the ability of the mutant strain to compete with the wild-type strain was assessed by determining the ratio of viable mutant cells to wild-type cells recovered after 24 h. The competitive index (the output ratio normalized to the input ratio) of the *fur* mutant was about 0.1, suggesting that the *fur* mutant is at a significant disadvantage for colonization of the small intestine (Fig. 3, group 1). Growing the strains under TCP-inducing conditions prior to inoculation did not yield a significantly different result (Fig. 3, group 2). To demonstrate complementation of the *fur* mutation with respect to in vivo colonization, the wild-type *fur* knock-in strain ARM574 was used as the competing strain against the *fur* mutant ARM573. This was done to avoid potential problems relating to inappropriate expression of *fur* from a plasmid or poor plasmid retention in vivo. As was seen

with the wild-type parental strain, the wild-type *fur* knock-in strain outcompeted the *fur* mutant strain in vivo (Fig. 3, group 3). Although the competitive index of the *fur* mutant was slightly higher in this experiment than in the competition with the wild-type strain O395, these results show that the reduced growth of the *fur* mutant in vivo is due, in large part, to the loss of Fur in this strain.

DISCUSSION

A number of recent studies have used microarray analysis to investigate the transcriptional response to changes in iron availability in wild-type or *fur* mutant bacteria (4, 17, 26, 36, 45, 55). Genes that were differentially regulated in these organisms generally included those involved in iron acquisition, iron storage and metabolism, stress response, and energy metabolism, as well as numerous ORFs of unknown function. A similar pattern of iron and Fur regulation was observed in this study. Sixty-five *V. cholerae* genes were identified that were derepressed in low iron and in the *fur* mutant. Forty-one of these have known roles in iron acquisition or iron metabolism. Of the remaining 24 genes, 11 were closely linked to known iron transport or metabolic genes and are likely controlled by the same elements that regulate those genes. Thus, only 14 novel genes negatively regulated by iron and Fur were identified in this study. Many of these novel genes have a potential Fur box in their promoter region (Table 4), and they may have as-yet-unidentified roles in iron acquisition or metabolism. Since nearly all of the known iron acquisition genes were identified in these arrays, and only a moderate number of additional genes were found, it is likely that Table 2 represents a nearly complete list of genes repressed by Fur and iron under the growth conditions used in this study. Besides classical iron-dependent repression by Fur, we observed several other regulatory patterns, including induction of gene expression by iron and Fur (Table 2), regulation by iron independently of Fur (Table 2), and regulation by Fur independently of iron (Tables 5 and 6). These latter results suggest that iron and Fur have certain nonoverlapping regulatory activities.

Iron regulation of genes not involved in iron acquisition could in some cases be anticipated based on their known roles in cellular metabolism or on the data from other organisms. Under low-iron conditions, cells alter gene expression to optimize the distribution of iron among their iron-containing proteins. For example, cells are protected from oxidative damage by the expression of superoxide dismutase (SOD) enzymes. *sodB*, encoding the iron-containing form of SOD, is repressed in response to iron limitation (12, 47), whereas *sodA*, encoding the manganese-containing form, is induced. This ensures sufficient SOD activity to protect the cell from oxidative stress when intracellular iron levels are low. Regulation of *sodA* by iron and Fur has been documented in several other species, including *E. coli* (52) and *Salmonella enterica* serovar Typhimurium (5). Similarly, *fumC*, which encodes the non-iron-containing form of the TCA enzyme fumarate hydratase, is induced in low iron, conditions under which the iron-containing form of the enzyme may be limited. Finally, genes such as the *nap* genes, which encode nonessential iron-containing proteins, may be repressed in low iron to increase the amount of iron available for the essential iron-containing enzymes.

In addition to competition for iron when this element is limiting, there may be competition for chorismate, an intermediate in the biosynthetic pathway of the siderophore vibriobactin. Because chorismate is needed also for the synthesis of other compounds, including aromatic amino acids, quinones, and folate, reducing the cell's dependence on some of these compounds may free up chorismate for vibriobactin synthesis. Indeed, we observed that *purN*, which encodes the formate-dependent form of the purine biosynthetic enzyme phosphoribosyl-*N*-formylglycinamide synthase, was upregulated in low iron, whereas *purT*, encoding the folate-dependent form of the same enzyme, was not. By upregulating the folate-free form when iron is limited, the need to allocate chorismate for folate production may be diminished, increasing the availability of chorismate for siderophore biosynthesis. In further support of this hypothesis, the menaquinone biosynthetic gene *menB* was negatively regulated in low iron. Since menaquinone is synthesized from chorismate, repressing its biosynthetic pathway may also increase the amount of chorismate available for siderophore biosynthesis.

The repression of *bfd* and *bfr* by iron and Fur is of note. Bfr is a ferritin-like protein containing both heme and iron, and Bfd is believed to be a ferredoxin that participates in either the loading or removal of iron from Bfr (21, 60). In *E. coli* (43, 45, 60, 68) and *Salmonella* (5), *bfd* is upregulated under low-iron conditions, whereas *bfr* is repressed. In *E. coli*, the repression of *bfr* is mediated by RyhB (43). These data suggest that iron starvation stimulates Bfd-dependent iron removal from Bfr while simultaneously limiting new synthesis of Bfr, which would no longer be needed under those conditions (43, 45, 60). However, the data in *V. cholerae* do not fit this model, since both *bfr* and *bfd* were upregulated in low iron. Further, there is no evidence that RyhB regulates *bfr* expression in *V. cholerae* (12, 47). Although Bfr contains iron and is generally considered to function in iron storage, this role is not strongly supported by genetic evidence, even in *E. coli* (1), and a greater understanding of the physiological role of Bfr will be required to resolve these differences.

Under iron-replete conditions, the Fur-Fe^{2+} complex inhibits expression of the negative regulator RyhB (12, 47). We therefore anticipated that, in low-iron medium and in the *fur* mutant, targets of RyhB repression would be significantly downregulated due to derepression of *ryhB* under those conditions. Surprisingly, none of the targets previously identified as being repressed by RyhB were affected in low iron or in the *fur* mutant. In fact, *ryhB* itself did not meet our criteria for inclusion in the list of genes induced in low iron during the early logarithmic phase, suggesting that factors other than iron and Fur may prevent induction of *ryhB* expression in this early growth phase. Similarly, we did not detect *ryhB* expression in the *fur* mutant under the conditions used for the microarray experiments (data not shown). *E. coli ryhB* is most strongly expressed in stationary phase (3), and *V. cholerae ryhB* is also highly expressed during the late logarithmic and early stationary phases (12, 47). In view of this, we must consider that other Fur-regulated genes, namely those with complex regulation involving additional factors, may not have been identified in these studies. Reports for other organisms have described a number of regulatory circuits that respond to changes in iron levels, including the stringent control pathways (72) and those

involving Crp (83) or the manganese regulator MntR (38, 40). These networks could potentially influence the iron-dependent Fur regulon in *V. cholerae*.

In *E. coli*, the nature of the Fur binding site has been the subject of some debate (reviewed in reference 2). Nonetheless, the Fur box consensus sequence for *V. cholerae* generated in this work is similar to the canonical sequence derived for *E. coli* (GATAATGAT[A/T]ATCATTATC). The similarity of the *E. coli* and predicted *V. cholerae* Fur box sequences is perhaps not surprising, since most of the *V. cholerae* Fur boxes were obtained by scanning the upstream regions of genes involved in iron uptake or metabolism for a match to the *E. coli* consensus. The close match between the *V. cholerae* and *E. coli* Fur box consensus sequences is supported by experimental data. The two *V. cholerae* Fur boxes that have been determined by DNase I footprinting have sequences very similar to the *E. coli* consensus (Table 3). In addition, reporter genes fused to the promoters of *V. cholerae* iron acquisition genes were regulated by iron in a Fur-dependent manner in *E. coli* (9, 34). A computer analysis screen for potential Fur binding sequences in the *V. cholerae* genome using a collection of known *E. coli* Fur boxes as the training set was moderately successful. Panina et al. (57) found potential Fur boxes upstream of 14 genes that were also regulated by Fur in this study; however, none of the chemotaxis genes identified in that study as having putative upstream Fur boxes was found to be regulated in our analyses.

There was considerable variability in the magnitude of regulation by iron and Fur among the genes studied here. Some of the most highly induced genes have more than one Fur box in their promoter region. These include the *tonB1*/*hutW* intergenic region (53, 80), the VCA0976 operon, *fumC*, and *irpA* (Table 3). The enterobactin receptor gene *irgA* was also strongly induced in low iron and in the *fur* mutant. Besides being repressed by Fur, *irgA* is positively regulated by IrgB, which is itself repressed by Fur (24, 76). This dual regulation may amplify the regulatory effects of iron and Fur. A similar situation may exist for the *vctA* and *hutA* genes (Table 2), which were greatly upregulated in low iron and in the *fur* mutant and are located adjacent to genes encoding potential positive transcription factors.

The *fur* mutant of *V. cholerae* exhibited a significant defect in the colonization of infant mice. Mutations in the *fur* gene of many other pathogenic species, including *Campylobacter jejuni* (55), *Listeria monocytogenes* (61), *H. pylori* (6), *Staphylococcus aureus* (37), *Actinobacillus pleuropneumoniae* (39), and *Bacillus cereus* (31), are associated with decreased virulence in animal models. In *C. jejuni*, a *fur* mutant exhibited significantly reduced colonization of the chick gastrointestinal tract. It was not clear, however, whether this was due to a generalized effect of the *fur* mutation on iron homeostasis or due to alterations in the expression of specific genes needed for the colonization process (55). Interestingly, the enterobactin transport system of *C. jejuni* is essential for in vivo growth (55), indicating that the chick gastrointestinal environment is iron restricted. This suggests that the *fur* mutant is probably not experiencing iron overload and iron toxicity in the in vivo environment, pointing to a more specific effect of the loss of Fur on colonization. An *H. pylori fur* mutant exhibited decreased gastric colonization in mice (6). This phenotype may be directly related to the lack of expression of the ferritin gene, *pfr*, in this strain, since *pfr*, which requires Fur for its expression (14), has been shown to be essential for colonization of the gastric mucosa of gerbils by *H. pylori* (73). In *L. monocytogenes*, the virulence defect of the *fur* mutant in mice could be overcome by overloading the mice with exogenous iron (61), suggesting that Fur is necessary for in vivo iron acquisition in this organism. In addition to its role in iron homeostasis, Fur controls the expression of a variety of bacterial toxins, and this may be critical for coordinating the synthesis of these important virulence factors with the appropriate in vivo signals (59). Thus, the role of Fur in pathogenesis is likely to be as multifaceted as its role in global iron regulation among these bacteria.

In *V. cholerae*, the virulence defect of the *fur* mutant may be linked to decreased production of the TCP, as measured by its autoagglutination defect. The autoagglutination phenotype is one indicator of the capacity of *V. cholerae* to establish an in vivo infection. Although the extent of autoagglutination does not correlate absolutely with the level of colonization, even moderately reduced autoagglutination may be associated with colonization defects (11). The results of the microarray analyses suggested that Fur may positively influence expression of multiple *tcp* genes, consistent with regulation by Fur of a central regulator in the *tcp* regulon. One such regulator could be the TcpPH complex, which positively regulates expression of *toxT* (10, 32). Because ToxT is a transcriptional activator of multiple genes in the *tcp* regulon (15), decreasing *tcpPH* expression would likely have a global effect on this group of genes. Although neither *tcpPH* nor *toxT* met our criteria for inclusion in the data set presented in Table 6, putative Fur binding sequences, matching up to 14 of the 19 Fur box consensus sequence nucleotides, have been identified in the *tcpI-tcpP* intergenic region (57), leaving open the possibility that Fur may directly control the transcription of either *tcpI* or *tcpPH*. By microarray analysis, the expression of *tcpI* was reduced in the *fur* mutant, but TcpI is not currently thought to be a positive regulator of the *tcp* cluster. In fact, TcpI has been proposed to function as a repressor of TCP expression, since a transposon insertion into *tcpI* allowed TCP expression under noninducing conditions (32). Thus, regulation of *tcpPH* by Fur presents a more attractive hypothesis and is currently under investigation.

No consistent effects on the expression of *tcp* genes were observed in microarray analyses of the iron-responsive transcriptome of *V. cholerae*, suggesting that the observed Fur-mediated regulation of the *tcp* regulon may be independent of iron. This could have implications for the manner in which Fur exerts its effects on gene expression in *V. cholerae*. A more careful analysis of multiple genes and/or operons regulated in this way might reveal promoter elements not previously associated with recognition by Fur.

ACKNOWLEDGMENTS

We thank Vishy Iyer and his laboratory for guidance and invaluable assistance with the microarray analyses and Amanda Oglesby, Erin Murphy, and Stephanie Craig for technical assistance and helpful discussions. We are also grateful to Mary Lozano for help with the mouse experiments.

This work was supported by the Foundation for Research and by National Institutes of Health grant AI50669.

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Editor: J. T. Barbieri